ORIGINAL RESEARCH

International Journal of Surgery 10 (2012) 96–101

Contents lists available at SciVerse ScienceDirect

International Journal of Surgery

journal homepage: www.theijs.com

Original research

The effect of hydroxyapatite-hPRP, and coral-hPRP on bone healing in rabbits: Radiological, biomechanical, macroscopic and histopathologic evaluation

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ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form
8 November 2011
Accepted 24 December 2011
Available online 8 January 2012

Keywords:
Persian Gulf coral
Hydroxyapatite
Radius
Bone healing
Rabbit

ABSTRACT

There is a continuing search for bone substitutes to avoid or minimize the need for autogenous bone grafts. Human platelet-rich plasma (hPRP) is used to stimulate bone formation *in vivo*. Hydroxyapatite, a crystalline phase of calcium phosphate found naturally in bone minerals, has shown tremendous promise as a graft material. Coral is an osteoconductive material used as a bone graft extender. This study examined the effect of human platelet-rich plasma in combination with hydroxyapatite and coral on osteogenesis *in vivo* using rabbit model bone healing.

A critical size defect of 10 mm elongation was created in the radial diaphysis of 36 rabbit and either supplied with human platelet-rich plasma (12 rabbits), and in combination with hydroxyapatite (12 rabbits), or coral (12 rabbits). Radiographs of each forelimb were taken postoperatively on 1st day and then at the 2nd, 4th, 6th and 8th weeks post injury to evaluate bone defect healing. The operated radiuses were removed on the 56th postoperative day and were grossly and histopathologically evaluated. In addition, biomechanical test was conducted on the operated and normal forearms of another half of the rabbits in each group. This study demonstrated that high concentrations of xenogenic platelets lead to superior and faster bone formation in comparison with hydroxyapatite-hPRP and coral-hPRP. Hydroxyapatite-hPRP and coral-hPRP resulted to almost similar results in bone healing process at this stage.

1. Introduction

Large bone defects resulting from trauma, tumors, osteitis, implant loosening or corrective osteotomies require surgical therapy, because spontaneous regeneration is limited to relatively small defects. Currently, transplantation of autografts or allografts, mineral bone substitutes and callus distraction are the most commonly used techniques for skeletal reconstruction, each of them having major limitations regarding availability, and biological or biomechanical reasons.[1,2] Therefore, osteoinductive stimulation of bone formation has received increasing interest.

Several investigations have previously demonstrated the positive effect of PRP on wound healing.[3–5] However, the results of these studies are controversial. In a bone defect in the iliac crest of dogs, PRP combined with demineralized bone powder enhanced bone formation around the titanium implants.[6] In a rabbit skull model, however, PRP did not influence bone healing.[7] There are numerous biomaterials available for use to promote bone healing,[8] but the exact indication of each of them remains controversial.

Hydroxyapatite, a crystalline phase of calcium phosphate found naturally in bone minerals, has shown tremendous promise as a graft material. It exhibits initial mechanical and structural rigidity, and demonstrates osteoconductive as well as angiogenic properties *in vivo*.[9] Calcium carbonate ($CaCO_3$) resembles hydroxyapatite in many respects. The material is biocompatible and osteoconductive but, similar to hydroxyapatite, has no osteoinductive properties.[10] The main difference of $CaCO_3$ with hydroxyapatite is its resorption rate.[11] The experiment was designed to compare the healing potential of hPRP delivered on a porous hydroxyapatite or coral with that of the hPRP alone as a third group on the healing of the long bone defects in a rabbit model.

2. Materials and methods

Thirty six New Zealand White rabbits, twelve-month-old of both sexes were kept in separate cages, fed a standard diet and allowed to move freely during the

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doi:10.1016/j.ijsu.2011.12.010

study. A critical size defect of 10 mm elongation was created in the radial diaphysis of 36 rabbit. In the animals of the hydroxyapatite-hPRP group (12 rabbits) and coral-hPRP group (12 rabbits), the bone defect was filled with hydroxyapatite segments (OS Satura®, Isotis Co, the Netherland) or natural coral [Coral exoskeleton from *Porites* sp. (Persian Gulf, Kish Island, Iran) was used in the form of cylindrical blocks of 10 mm long and 4 mm in diameter. The coral implants were sterilized by autoclaving so that the composition remained intact[12]] segments, respectively. In hPRP group the defect was filled only by hPRP. Four days after operation 1 ml hPRP (Human PRP was prepared and supplied by the Shiraz Blood Bank Center, Number of platelets in the whole blood and PRP was 239×10^9/l and 2422×10^9/l respectively.) was injected percutaneously into the defect of bones in the animals of all three groups. The animals were housed in compliance with our institution's guiding principles "in the care and use of animals". The local Ethics Committee for animal experiments approved the design of the experiment.

To radiological evaluation of the defect, radiographs of each forelimb were taken postoperatively on 1st day and then at the 2nd, 4th, 6th and 8th weeks post injury. The results were scored using the modified Lane and Sandhu scoring system[13] (Table 1). The sum of bone formation, proximal union, distal union and remodeling scores were analyzed and compared between groups at the 2nd, 4th, 6th and 8th weeks post injury intervals.

The operated radial bones were removed on 56th postoperative day; at this time the operated radius was evaluated for gross signs of healing. Examination and blinded scoring of the specimens included presence of bridging bone, indicating a complete union (+3 score), presence of cartilage (+2 score), soft tissue or cracks within the defect indicating a possible unstable union (+1 score), or complete instability at the defect site indicating no union (0 score).

The histopathological evaluation was carried out on six rabbits of each group randomly. The sections were stained with hematoxylin and eosin and blindly scored by two pathologists according to the Emery's scoring system.[14] Based on this scoring system the defects were evaluated as follows: when the gap was empty (score = 0), if the gap was filled with fibrous connective tissue only (score = 1), with more fibrous tissue than cartilage (score = 2), more cartilage than fibrous tissue (score = 3), cartilage only (score = 4), more cartilage than bone (score = 5), more bone than cartilage (score = 6) and filled only with bone (score = 7).

The biomechanical test was conducted on the injured and normal contralateral bones of six other rabbits of each group. The tests were performed using a universal tensile testing machine (Instron, London, UK).[15–17] The three-point bending test was performed to determine the mechanical properties of bones.

The radiological, clinical and histopathological data were compared by Kruskal–Wallis, non-parametric ANOVA, when *P-values* were found to be less than 0.05, then pair wise group comparisons was performed by Mann–Whitney U test. The biomechanical data were compared by a student's *t-test* between the treated and normal limb data and one way ANOVA test was used for biomechanical analysis between the treated bones of all groups (SPSS version 17 for Windows, SPSS Inc, Chicago, USA).

3. Results

3.1. Radiological findings

There was radiologically a significant difference in healing of the bone defect between hPRP group with those of the hydroxyapatite-hPRP and coral-hPRP treated ones on the 14th post-injury day. Healing of the bone defect in the animals of the hPRP group was superior to those of the hydroxyapatite-hPRP or coral-hPRP ones. There were no significant radiological differences in healing of the bone defect between the animals of all three groups on 28th and the 42nd post-injury day. There was only a significant difference in the healing of the bone defect between the animals of the hPRP group with those of the coral-hPRP rabbits on the 56th post-injury day (Table 2, Figs. 1–3).

Table 1
Modified Lane and Sandhu radiological scoring system.

Bone formation	
No evidence of bone formation	0
Bone formation occupying 25% of the defect	1
Bone formation occupying 50% of the defect	2
Bone formation occupying 75% of the defect	3
Bone formation occupying 100% of the defect	4
Union (proximal and distal evaluated separately)	
No union	0
Possible union	1
Radiographic union	2
Remodeling	
No evidence of remodeling	0
Remodeling of medullary canal	1
Full remodeling of cortex	2
Total point possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum Score	10

Table 2
Radiographical findings for bone defect healing (sum of radiological scores) at various postoperative intervals.

Postoperative days	Med (min–max)			P^a
	Hydroxyapatite-hPRP ($n = 12$)	Coral-hPRP ($n = 12$)	hPRP ($n = 12$)	
14	1(0–2)	0(0–2)	2(1–4)[b,c]	0.006
28	3(1–7)	3(0–5)	5(2–8)	0.14
42	7(3–8)	5(3–7)	8(2–9)	0.17
56	8(4–10)	7(4–8)	9(4–10)[d]	0.05

Significant *P*-values are presented in bold face.
[a] Kruskal–Wallis non-parametric ANOVA.
[b] $P = 0.01$ (compared with Hydroxyapatite-hPRP by Mann–Whitney U test).
[c] $P = 0.004$ (compared with Coral-hPRP by Mann–Whitney U test).
[d] $P = 0.02$ (compared with Coral-hPRP by Mann–Whitney U test).

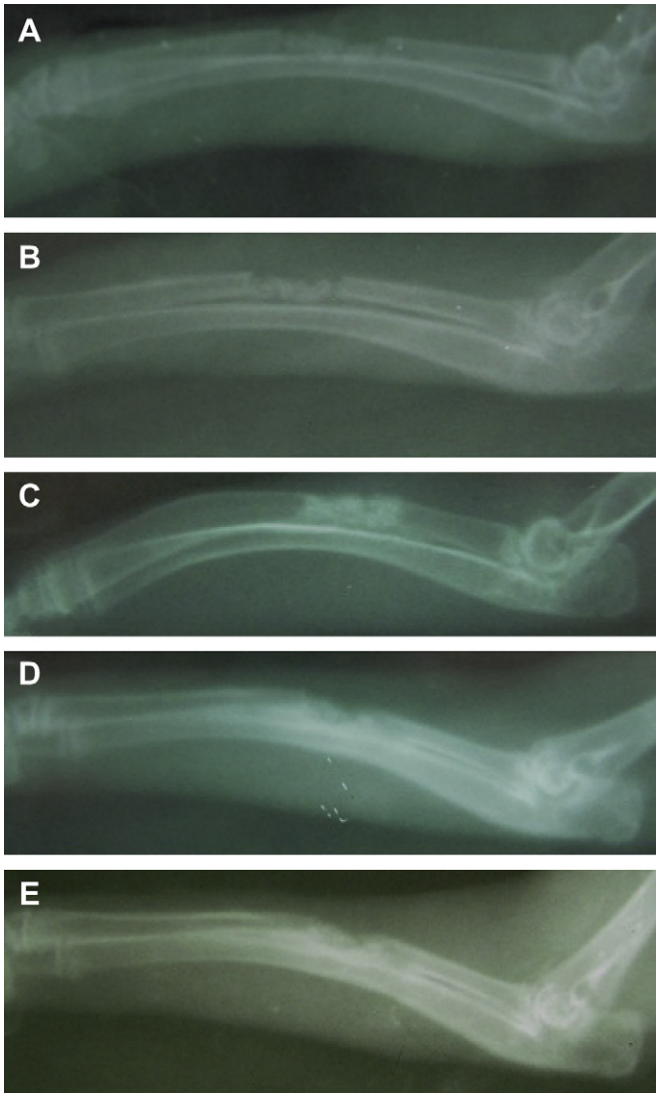

Fig. 1. Radiographs of treated forelimb in hydroxyapatite-hPRP group, on 1st day (A), 14th postoperative day (B), 28th postoperative day (C), 42nd postoperative day (D) and 56th postoperative day (E).

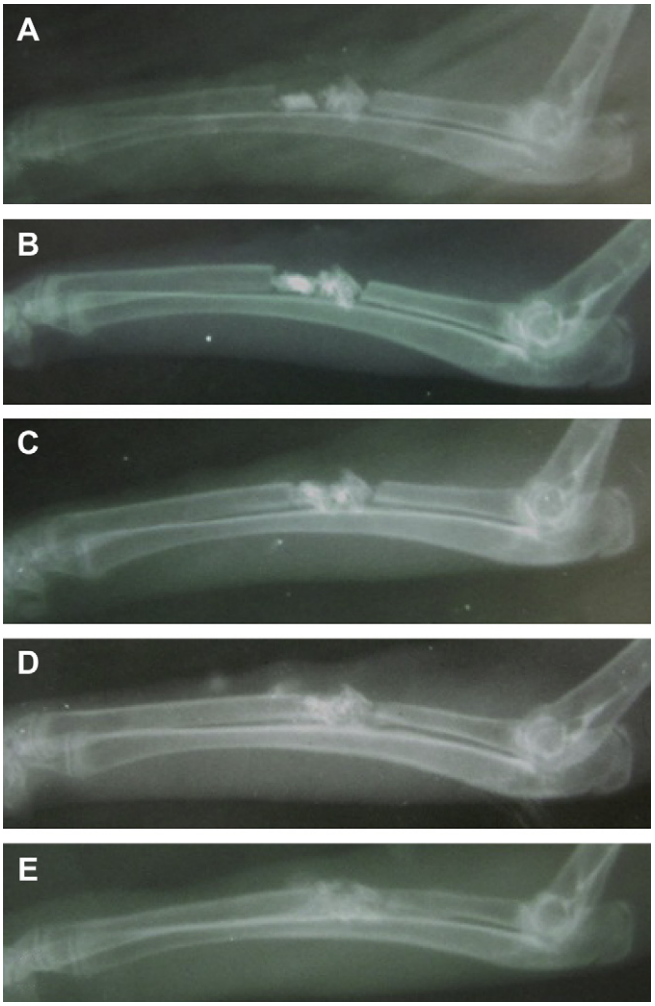

Fig. 2. Radiographs of treated forelimb in coral-hPRP group, on 1st day (A), 14th postoperative day (B), 28th postoperative day (C), 42nd postoperative day (D) and 56th postoperative day (E).

3.2. Gross and histopathological findings

The defect areas of the rabbits in all groups showed various amounts of new bone formation. The union scores of the rabbits administered with hPRP or hydroxyapatite-hPRP or coral-hPRP were not statistically different ($P = 0.3$, Table 3). The union scores at macroscopic level correlated closely with the radiographic union score on day 56 post injury. In all cases, the defect area generally contained various amount of new bone that in most instances were filled with a mixture of bone and cartilage.

At histopathologic level, the defects of the animals of all three groups showed proper healing criteria without any statistically significant differences ($P = 0.4$, Table 3, Fig. 4). No significant inflammatory response was evident in the lesions of the animals of different groups at 8 weeks post injury, although it may have been present earlier.

3.3. Biomechanical findings

The injured leg of all animals in all groups showed proper biomechanical properties so that there was no statistically significant difference in the ultimate strength, stiffness, stress and strain between the normal and treated limb of the animals of the three different groups ($P > 0.05$) and between the treated limbs in all groups ($P = 0.4$, Table 4) on 56th days post injury.

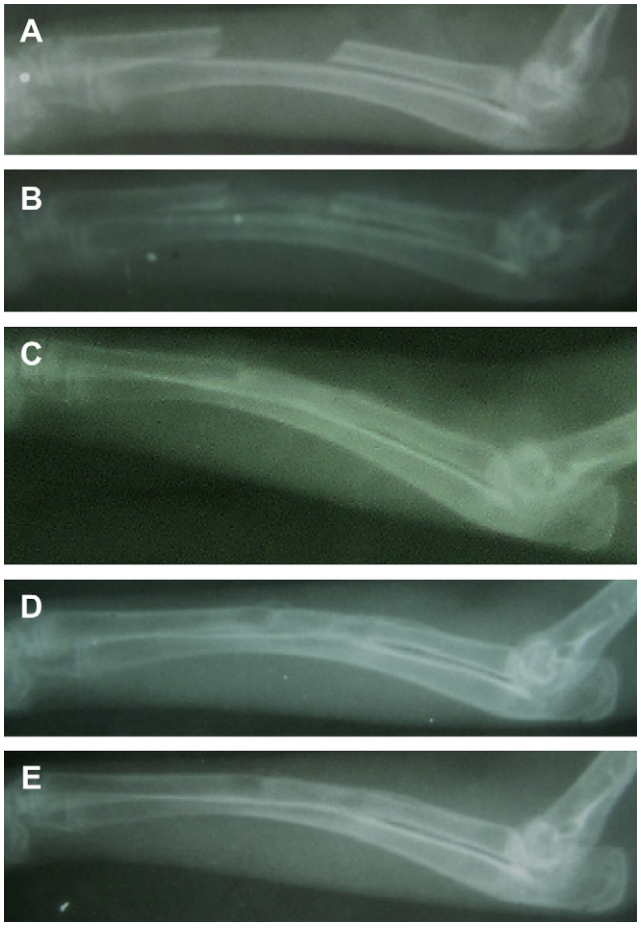

Fig. 3. Radiographs of treated forelimb in hPRP group, on 1st day (A), 14th post-operative day (B), 28th postoperative day (C), 42nd postoperative day (D) and 56th postoperative day (E).

4. Discussion

The objective of this study was to evaluate the healing of a critical-sized radial bone defect treated with hPRP and compare it with hydroxyapatite or natural coral in combination with hPRP. The radial bone defect of rabbits is a convenient model for study of bone-regenerative materials because of its lack of fixation requirements.[18] Small rodents have primitive bone structures and do not have haversian systems[19] and although little is known about the importance of this anatomical difference between rodents and humans, this makes bone repair in these animals different from that seen in human beings.[19] Whereas rabbits, as well as caprines and dogs, have haversian systems that are similar to that of human, which is an important advantage in terms of extrapolation of results obtained with such animals for human bone repair.[19] However, the rapid healing processes in these models compared with humans, make them a valuable bioassay for screening of comparable technologies, but questionable for direct transfer of information to the human clinical situation.[20]

The radiological results showed that bone healing was enhanced when hPRP was used alone in comparison with hydroxyapatite-hPRP or coral-hPRP. These results are not in agreement with those of Mooren et al. (2007) because they showed that the goat PRP was not able to enhance early or late bone healing in a goat skull bone healing model.[21] In addition, our radiological results are not in agreement with Aghaloo et al. (2002) results,[7] because they showed a significant increase in radiographic bone density in both

Table 3
Bone measurements at macroscopic and microscopic level.

Bone type evaluation	Med (min–max)			P^a
	hPRP-hydroxyapatite ($n = 6$)	Coral-hPRP ($n = 6$)	hPRP ($n = 6$)	
Macroscopic union[b]	3 (2–3)	2 (1–3)	3 (1–3)	0.3
Microscopic evaluation[c]	7 (6–7)	6 (5–7)	7 (6–7)	0.4

Significant *P*-values are presented in bold face.
[a] Kruskal–Wallis non-parametric ANOVA.
[b] Complete union (+3 score), presence of cartilage, soft tissue or cracks within the defect indicating a possible unstable union (+1 or +2 score), complete instability at the defect site indicating nonunion (0 score).
[c] Empty (0 score), fibrous tissue only (1 score), more fibrous tissue than fibrocartilage (2 score), more fibrocartilage than fibrous tissue (3 score), fibrocartilage only (4 score), more fibrocartilage than bone (5 score), more bone than fibrocartilage (6 score) and bone only (7 score).

bone and bone-PRP samples as compared with the control and PRP alone in a rabbit model. However, there were no significant differences in different macroscopical, histological and biomechanical criteria of the animals of all groups in our study.

The clinical and experimental data in the literature regarding the osteogenic potential of PRP are controversial. The results of the present investigation confirm a number of clinical and experimental studies demonstrating a positive influence of hPRP on bone regeneration.[22–25] However, in human maxillofacial defects, neither the autograft nor allograft or the mineral bone substitute material enhanced bone formation when augmented with PRP.[26–28] In a non-critical rabbit skull defect, autogenously PRP was not superior to the empty defect nor did PRP increased bone formation by autogenous bone.[7]

This study demonstrated the hPRP's role in treating bone defects. From the radiological measurements analyses described in

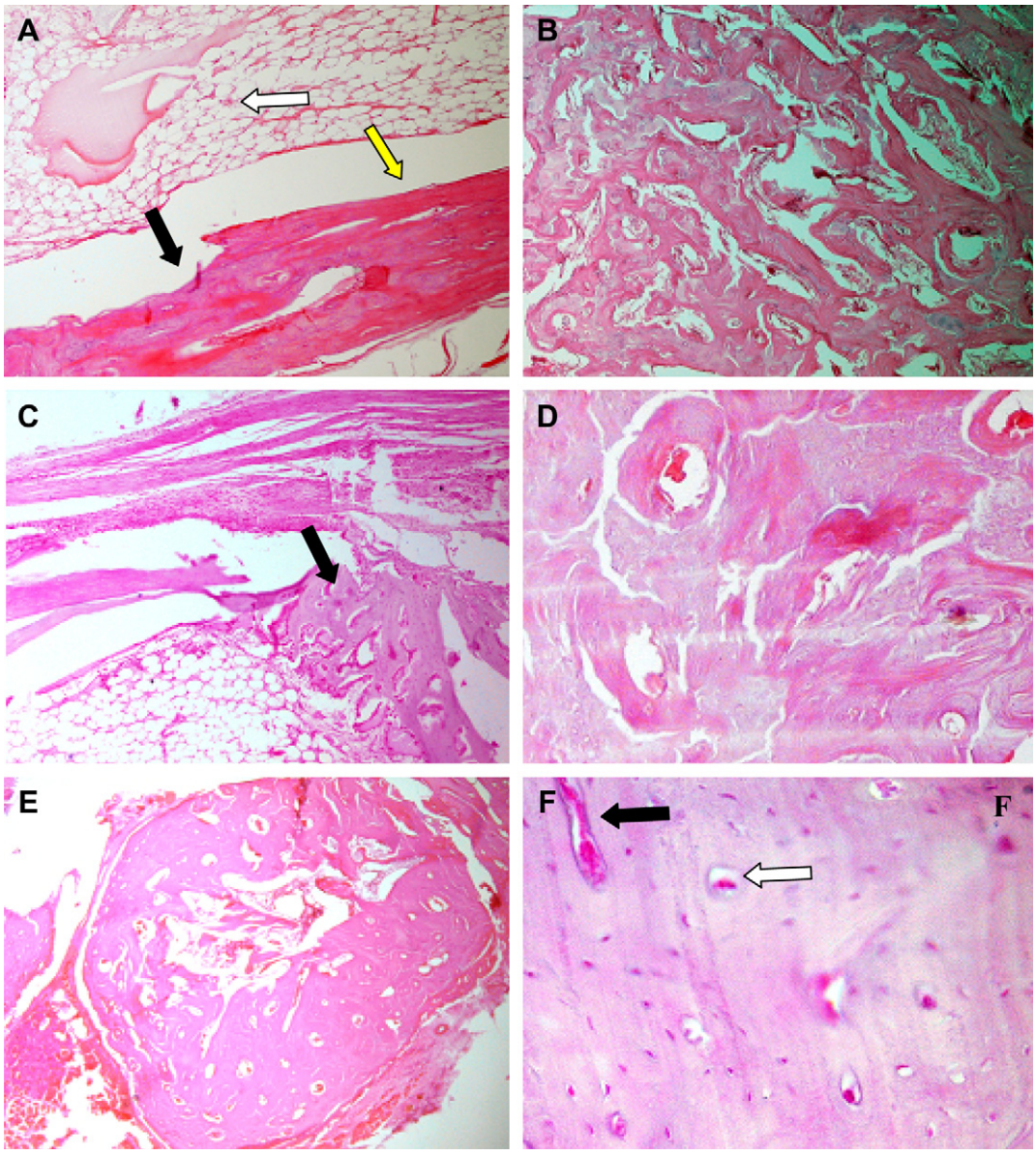

Fig. 4. Photomicrograph of hydroxyapatite-hPRP group on the 56th postinjury, grafted hydroxyapatite (black arrow) was remodeled and bone marrow (white arrow) was formed, note to old bone (yellow arrow) and remodeled marrow in the grafted region (A, H & E stain 10x). Note to extensive trabecular bone in the grafted area (B, H & E stain 10x). Photomicrograph of coral-hPRP group, note to grafted region, woven bone formation (black arrow) without bone marrow remodeling (C, H & E stain 4x) and note to bone-cartilage tissues in the grafted area (D, H & E stain 10x). Photomicrograph of hPRP group, compact cortical bone and marrow formation was observed in grafted area (E, H & E stain 4x). Note to compact bone formation with several Haversian canal (white arrow) and Volkmann canal (black arrow) (F, H & E stain 40x).

Table 4
Biomechanical findings after 56th postoperative day.

Three point bending test criteria	Mean ± SEM					
	hPRP-hydroxyapatite ($n = 6$)		Coral-hPRP ($n = 6$)		hPRP ($n = 6$)	
	normal limb	treated limb	normal limb	treated limb	normal limb	treated limb
Ultimate Strength (N)	(108.0 ± 17.2)	(95.0 ± 12.3)	(83.5 ± 17.6)	(74.33 ± 5.8)	(98.6 ± 7.7)	(99.1 ± 19.1)
Stress (N/mm^2)	(6.5 ± 0.9)	(4.3 ± 0.8)	(4.9 ± 0.8)	(4.2 ± 0.7)	(6.08 ± 0.77)	(6.28 ± 0.69)
Stiffness (N/mm)	(76.6 ± 13.08)	(83.3 ± 11.7)	(91.6 ± 11.6)	(90.0 ± 28.2)	(118.3 ± 14.4)	(105.0 ± 5.0)
Strain (%)	(5.8 ± 0.4)	(6.6 ± 0.80)	(8.08 ± 0.4)	(7.4 ± 0.6)	(8.52 ± 0.4)	(8.1 ± 0.1)

this study, significant differences were present between the defects of the animals of the hPRP treated group with those of the two other groups.

The platelet-rich plasma contains several growth factors including isomers of platelet derived growth factor (PDGF), transforming growth factor-X1 (TGF-X 1), transforming growth factor-2 (TGF-2), Insulin like growth factor-I (IGF-I), Insulin like growth factor-II (IGF-II) and vascular endothelial growth factor (VEGF). All these growth factors are promoters of bone regeneration. The platelet derived growth factor has been shown to be mitogenic for osteoblasts[29] and stimulates migration of the mesenchymal progenitor cells.[30] It has been stated that PDGF was able to induce callus formation in the bone defects of the animal models.[31] TGF-X also has a stimulative effect on osteogenesis and inhibits bone resorption.[32] In addition, it has been reported that IGF-I and the angiogenic factor VEGF induced bone formation in rats[33] and rabbits.[34] The findings of the present study suggest that the superiority of hPRP in combinations with the other two types of biomaterial has possibly been due to the presence of VEGF in human platelet. However, in the two other groups it is possible that the effects of hPRP have been obscured with by hydroxyapatite or coral, so that angiogenesis in the defects of the animals of these two groups were inferior to those of the hPRP ones.

These growth factors support bone regeneration primarily via their chemotactic and mitogenic effects on preosteoblastic and osteoblastic cells. Due to this phenomenon, enhanced bone formation criteria in the defects of the animals of the hPRP group compared to those of the other two groups were observed. However, hPRP does not contain BMPs, the most potent osteoinductive proteins, that are the only growth factors known to induce ectopic bone formation which promote stem cells to differentiate into the osteoblastic lineage.[35] However, in the present study, after 56 days, the hPRP group did not show any significant differences with other two groups in biomechanical, macroscopical and histopathological criteria. The authors proposed that there might be some differences at the earlier stages of the healing but by 8 weeks post injury they reached to almost level.

The enhanced healing effects of the hPRP after combination with human bone graft material, compared to a combination with a synthetic bone substitute, can also be explained by the mechanism of action of PRP. According to Marx et al.,[36] PRP is thought to exert its effects on living cells. Consequently, when PRP is used together with synthetic, non-cellular bone substitutes less promotion of bone formation could be expected compared to its application with the bone graft material. The beneficial effects of PRP applied in combination with a synthetic bone substitute, depend on the number of the resident osteoprogenitor cells at the bone defect site. Occasionally, the osteoconductive materials can obscure the true effects of PRP. In the present study, combination of hPRP with hydroxyapatite or natural coral did not lead to superior bone healing in comparison with hPRP alone. Therefore, based on the findings of the present study, it could be concluded that even high concentrations of platelets in combination with hydroxyapatite or coral is not effective and did not lead to superior and faster bone formation. However, high concentrations of xenogenic platelets in the present study lead to superior and faster bone formation. While Schlegel et al.[24] and Thorwarth et al.[25] got better results by administering higher doses of hPRP (6.5-fold compared to normal blood) than with lower platelet concentrations (4.1-fold) on bone regeneration in skull defects of minipigs,[24,25] some other experimental studies found no correlation between the platelet concentration and the observed biological effects.[6,7]

In the present study hydroxyapatite-hPRP was superior to coral-hPRP in radiological evaluation. However, on day 56th post injury they were almost similar from the histopathological or biomechanical stand points. While, the previous *in vitro* studies have shown that artificial bone graft materials supports the attachment, growth and differentiation of the bone-marrow stromal cells.[37]

5. Conclusion

In conclusion this study demonstrated that high concentrations of xenogenic platelets lead to superior and faster bone formation and after 8 weeks post injury hydroxyapatite-hPRP and coral-hPRP redound to bone healing in a similar condition.

Conflict of interest statement

There are no conflicts of interest related to this study.

Funding

The source of funding for this study was the Grant of Research Council of Shiraz University.

Ethical approval

The local Ethics Committee for animal experiments approved the design of the experiment.

Author contribution

Prof Meimandi, Dr. Z Shafiei-Sarvestani, Prof Oryan and Dr. Bigham Sadegh were involved in all study procedures such as study design, data collections, data analysis and writing.

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